

# International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies

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**Handling editor** Tore K Kvien

► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/annrheumdis-2013-203863>).

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Received 30 April 2013

Revised 9 August 2013

Accepted 20 September 2013

## ABSTRACT

Anti-nuclear antibodies (ANA) are fundamental for the diagnosis of autoimmune diseases, and have been determined by indirect immunofluorescence assay (IIFA) for decades. As the demand for ANA testing increased, alternative techniques were developed challenging the classic IIFA. These alternative platforms differ in their antigen profiles, sensitivity and specificity, raising uncertainties regarding standardisation and interpretation of incongruent results. Therefore, an international group of experts has created recommendations for ANA testing by different methods.

Two groups of experts participated in this initiative. The European autoimmunity standardization initiative representing 15 European countries and the International Union of Immunologic Societies/World Health Organization/Arthritis Foundation/Centers for Disease Control and Prevention autoantibody standardising committee. A three-step process followed by a Delphi exercise with closed voting was applied.

Twenty-five recommendations for determining ANA (1–13), anti-double stranded DNA antibodies (14–18), specific antibodies (19–23) and validation of methods (24–25) were created. Significant differences between experts were observed regarding recommendations 24–25 ( $p < 0.03$ ). Here, we formulated recommendations for the assessment and interpretation of ANA and associated antibodies. Notably, the roles of IIFA as a reference method, and the importance of defining nuclear and cytoplasmic staining, were emphasised, while the need to incorporate alternative automated methods was acknowledged. Various approaches to overcome discrepancies between methods were suggested of which an improved bench-to-bedside communication is of the utmost importance. These recommendations are based on current knowledge and can enable harmonisation of local algorithms for testing and evaluation of ANA and related autoantibodies. Last but not least, new more appropriate terminologies have been suggested.

## INTRODUCTION

Autoantibodies are a hallmark of autoimmunity, of which anti-nuclear antibodies (ANA) have taken the centre stage for the past 60 years. The term ANA is now outdated and even confusing as this historical label has come to encompass antibodies directed at various cellular compartments including nuclear constituents, components of the nuclear envelope, mitotic spindle apparatus, cytosol, cytoplasmic organelles and cell membranes. Detection of anti-cellular antibodies of the ANA family is pivotal to the diagnosis of many autoimmune diseases.<sup>1–2</sup> Moreover, specific antibodies of the ANA family may present years before the appearance of overt disease, and for some conditions serological assays can provide useful information on the likelihood of clinical course or complications (eg, inflammatory myopathies, systemic lupus erythematosus (SLE)). Hence, the determination of ANA may enable the prediction, diagnosis and activity determination of certain autoimmune diseases.<sup>3–4</sup>

ANA detection by the indirect immunofluorescence assay (IIFA) was first described in 1950 by Coons and Kaplan.<sup>5</sup> This ‘gold’ standard technique is at present performed using HEp-2 cells or variants of this cell line (ie, HEp-2000).<sup>6</sup> The IIFA requires a laborious process consisting of serial dilutions of positive sera, visual determination of the staining pattern, followed by a second test in which antigen specificity is determined.<sup>7–8</sup> IIFA entails substantial technical expertise and while it is considered the ‘gold’ standard, it is only as good as the laboratory that performs this assay. Another limitation of IIFA is its lack of specificity. Indeed, depending on demographics, the population being studied, serum dilution, the cut-off used and other variables of this assay, up to 25% of sera from apparently healthy individuals can be ANA positive.<sup>9–14</sup> Noteworthy, in the general population some individuals with a positive ANA test by IIFA do not have an autoimmune disease and are unlikely to develop one.

**To cite:** Agmon-Levin N, Damoiseaux J, Kallenberg C, et al. *Ann Rheum Dis*. Published Online First: [please include Day Month Year] doi:10.1136/annrheumdis-2013-203863

## Recommendation

In past decades, the demand for ANA testing has increased remarkably, raising the need for high throughput in service laboratories. These changes resulted in a technical evolution and the development of novel diagnostic platforms including the ELISA, addressable laser bead immunoassay, chemiluminescence immunoassay, fluorescent-enzyme immunoassay, line immunoassays<sup>10</sup> and others.<sup>8 15 16</sup> The latter are based on a limited number of purified and/or synthetic autoantigens. For details on the methodology see the supplementary material (available online only). The test characteristics of these novel assays are strongly distinct from the ANA-IIFA. Debates regarding ANA determination by different tools have recently resulted in a position statement of the American College of Rheumatology (ACR), claiming that IIFA is the 'gold' standard for ANA detection, and that alternative methods should demonstrate performance as good as IIFA ([http://www.rheumatology.org/practice/clinical/position/ana\\_position\\_stmt.pdf](http://www.rheumatology.org/practice/clinical/position/ana_position_stmt.pdf)). This position statement is primarily based on the high sensitivity of IIFA for certain autoimmune diseases such as SLE and to a lesser extent systemic sclerosis (SSc). Moreover, it is strongly supported by the historical inclusion of the ANA, as performed by IIFA, as a criterion of certain diseases. Unfortunately, IIFA specificity and sensitivity is relatively low for other diseases (eg, inflammatory myopathies) and its technical limitations as detailed above are yet to be overcome.

Therefore, an international group of experts (laboratory specialists, scientists and clinicians) have developed a set of recommendations for the appropriate assessment and interpretation of anticellular antibodies of the ANA family determined by different methods.

## METHODS

### Expert committees

Two groups of experts participated in this initiative. The European autoimmunity standardization initiative (EASI) was formed a decade ago. This forum was assembled with the aims of improving the diagnosis of systemic autoimmune-rheumatic diseases (SARD), the rationale of autoantibody testing and to stimulate interactions between clinical and laboratory specialist while considering cost-effectiveness and novel techniques (<http://www.easi-network.com/>). In this study expert teams from 15 European countries, namely Italy, Germany, The Netherlands, France, UK, Sweden, Belgium, Finland, Norway, Switzerland, Austria, Spain, Ukraine, Portugal and Israel participated. Each national EASI team consists of clinicians, laboratory specialists and scientists, dedicated to the field of SARD. The final vote for each team was the average score of all participants from the same country.

The International Union of Immunologic Societies/World Health Organization/Arthritis Foundation/Centers for Disease Control and Prevention (IUIS/WHO/AF/CDC) committee for the standardisation of autoantibodies in rheumatic and related diseases (<http://www.autoab.org>) was established in the early 1980s, and is also affiliated with the ACR. The establishment of this group was primarily based on the recognised needs for reference human autoimmune sera that were critical for academic, clinical and industrial laboratories, as well as the need to communicate to the wider stakeholder community and to facilitate ongoing activities in continuing the mission in autoantibody standardisation.<sup>17</sup>

### Development of recommendations

A three-step process was applied for creating these recommendations for the determination and interpretation of ANA and

related antigen-specific antibody testing. First, 31 preliminary statements were generated by the EASI forum, based on previous algorithms from Italy, Germany, The Netherlands and the ACR. Following review of the literature, these recommendations were subjected to open comments by the EASI teams and grading by each EASI team on a scale of 1–5 (ie, full agreement to no agreement, respectively). Thereafter, based on the results of the first stage, the EASI forum reformulated a new set of 25 recommendations. These were subjected to review and modifications by both the EASI teams and the IUIS/WHO/AF/CDC committee members.

In the third stage a Delphi exercise with closed voting followed. During this exercise the final 25 recommendations were separately voted on and given a score from 0 (absolutely no agreement with the recommendation) to 10 (maximal support for the recommendation). The means and SD scores of all participants were calculated to determine the level of agreement for each recommendation. Therefore, the highest agreement is defined by the highest mean score accompanied by the lowest SD. Separate scores for the voting by the EASI groups versus the IUIS members were assessed using the t test ( $p < 0.05$  was considered significant).

## RESULTS

Twenty-five recommendations were formulated in this study and divided into four subgroups. The first 13 recommendations were dedicated to the ANA test (1–13), five to the determination of anti-double stranded DNA antibodies (14–18) and five to the determination of specific antibodies including those directed to extractable nuclear antigens (ENA; 19–23). Recommendations 24–25 addressed the need for local validation of different methods. Each recommendation was graded by a Delphi voting score composed of voting by the EASI and the IUIS groups. Scores are presented as the mean  $\pm$  SD of the entire forum (table 1).

Significant statistical differences between the voting of the EASI teams and the IUIS members were observed only regarding recommendations 24 and 25, which received the grades  $8.3 \pm 3$  vs  $10 \pm 0$  and  $7.4 \pm 2.9$  vs  $10 \pm 0$ , respectively ( $p < 0.03$ ).

The broad spectrum of nuclear and cytoplasmic staining patterns, in relation to antibody specificity and associated disease, is specified in table 2.

## DISCUSSION

In this study we formulated 25 recommendations intended to clarify and standardise clinical and technical aspects pertaining to the determination of ANA and related antigen-specific antibodies. In addition, key principles regarding terminology, discrepancies between methods and communication between laboratory and clinical specialists were addressed.

### Terminology

The terms 'anti-nuclear antibodies' (ANA) and 'extractable nuclear antigens' (ENA) are no longer technically correct and do not cover the entire spectrum of relevant autoantibodies. 'ANA' may now detect antibodies directed against nuclear and non-nuclear elements (see recommendation 13), while 'ENA' may refer to antigens that are neither extractable nor nuclear. Therefore, one may suggest changing these outdated terms to appropriate ones, such as anticellular antibodies and specific antibodies, respectively. Such a change in nomenclature requires broad agreement within the medical community and an adjustment period, as most manuscripts and textbooks utilise these 'classic terms'. In the current manuscript we have used the

**Table 1** The recommendations for detection of autoantibodies to cellular components commonly referred to as ANA

	Recommendation	Delphi score (mean±SD)
1	The diagnosis of SARD requires a panel of specific laboratory tests (ie, ANA, anti-dsDNA and anti-ENA antibodies)	9.6±0.9
2	ANA, anti-dsDNA and anti-ENA testing should be included in the autoantibodies detection as part of the diagnostic work-up of SARD as well as some other autoimmune diseases (table 2)	9.9±0.2
3	The detection of ANA is the first level test for laboratory diagnosis of SARD	9.8±0.4
4	ANA testing is primarily intended for diagnostic purposes, and not for monitoring disease progression	9.6±0.6
5	The IIFA* is the reference method for ANA screening. Alternative assays can be used while keeping in mind that false negative and false positive ratio of these methods may be different. Thus, if the clinical suspicion is strong and the alternative method is negative, it is mandatory to perform IIFA	9.7±0.6
6	Diagnostic laboratories should specify the methods used for detecting ANA when reporting their results	9.4±0.9
7	Tests based on a (restricted) mixture of defined nuclear antigens should not be referred to as ANA test or ANA screen	9.7±0.5
8	Laboratories using in-house assays for detecting ANA, as well as anti-dsDNA and specific anti-ENA antibodies, should standardise each assay according to international standards (eg, WHO, CDC/IUIS)	9.7±0.5
9	For ANA screening by IIFA the conjugate should consist of fluorochrome-labelled anti-human IgG-specific secondary antibodies	9.2±1.2
10	A proper ANA–IIFA is dependent on reagents, equipment and other local factors, thus the screening dilution should be defined locally. An abnormal ANA should be the titre above the 95th percentile of a healthy control population. In general, a screening dilution of 1 : 160 on conventional HEp-2(000) substrates is often suitable for the detection of ANA in adult populations being evaluated for SARD	9.0±1.4
11	In the case of a positive ANA test, it is recommended that the pattern and the highest dilution to demonstrate reactivity be reported	9.4±0.9
12	ANA–IIFA patterns should be reported according to standardised terminology (table 2)	9.7±0.4
13	Besides nuclear patterns also cytoplasmic and mitotic apparatus patterns should be reported and specified when possible (table 2)	9.5±1.0
14	If ANA result is positive, testing for anti-dsDNA antibodies is advised when there is clinical suspicion of SLE	9.7±0.4
15	For anti-dsDNA antibody determination, the Farr assay and the CLIFT offer high clinical specificity. Alternative methods may yield lower specificity and, if so, it is recommended that positive results obtained by these methods be confirmed by CLIFT or Farr assay—and be reported separately	8.0±2.5
16	The method used for anti-dsDNA antibody detection should be included in the test result	9.4±0.7
17	Results of anti-dsDNA antibody detection should be reported quantitatively (or semiquantitatively for CLIFT)	9.7±0.4
18	For monitoring of SLE disease activity by quantitative determination of anti-dsDNA antibodies the same method should be used	9.9±0.3
19	In case of a positive ANA test during the diagnostic work-up (depending on pattern, titre and/or clinical setting), it is recommended to perform specific tests for anti-ENA antibodies (table 2)	9.8±0.3
20	For anti-ENA antibodies detection the method used should be reported. In the case of discrepancy with IIFA or with clinical suspicion, the use of an additional method should be considered	9.5±0.5
21	Results of assays for antibodies to specific ENA should be reported separately (including negative results); if the result of a screening assay is reported as negative, it is sufficient to communicate which ENA are present in that assay	9.8±0.4
22	Quantitative determination of positive anti-RNP antibodies is recommended in case of a clinical suspicion of mixed connective tissue disease	8.2±2.7
23	In case of high clinical suspicion the physician request for determination of antibodies to specific ENA should be granted, irrespective of the result of the ANA test. For instance, anti-Jo-1 antibodies for clinically suspected IM, anti-ribosomal P for SLE or anti-SS-A/Ro antibodies for congenital heart block/neonatal lupus/Sjögren's syndrome/subacute cutaneous lupus	9.9±0.1
24	Each laboratory should verify the recommended cut-off for kits used to determine ANA. It is recommended to use age and gender matched sera from healthy subjects from the general local population; cut-offs should be defined as the 95th percentile	8.4±2.8
25	Each laboratory should verify the recommended cut-off for kits used to determine anti-dsDNA and anti-ENA antibodies. It is recommended to use an adequate number of samples from patients with the appropriate autoimmune diseases, disease controls and healthy controls; cut-offs should be defined using ROC curve analysis	7.6±2.9

\*See supplementary materials (available online only) for further information on the methodology of immunoassays.

ANA, anti-nuclear antibodies; CDC, Centers for Disease Control and Prevention; CLIFT, *Crithidia luciliae* immunofluorescence test; dsDNA, double-stranded DNA; ENA, extractable nuclear antigens; IIFA, indirect immunofluorescent assay; IM, inflammatory myopathies; IUIS, International Union of Immunologic Societies; RNP, ribonucleoproteins; ROC, receiver operating characteristic; SARD, systemic autoimmune rheumatic diseases; SLE, systemic lupus erythematosus; WHO, World Health Organization.

traditional term ANA and ENA, with the appreciation that they refer to a broader antigen specificity.

### Discrepancy between platforms

The increasing demand for ANA tests led to the development of new automated methods for their detection, some of which probably represent the future of diagnostic serology.<sup>18</sup> However, the inconsistencies between methods are currently a burden on those who perform and interpret these tests. For this reason, we have addressed the concepts of discordant results and communication between laboratory and clinical specialists. As a general rule, if discrepancy between methods is observed, especially in the setting of high clinical suspicion, another approved and validated diagnostic platform should be utilised. Notably, in certain cases, choosing another platform or deciding which method is preferred may require a collaborative assessment of clinicians and laboratory specialists.<sup>19</sup> For example, the use of

anti-dsDNA antibody titration for monitoring SLE activity is better achieved by using a quantitative assay such as ELISA or the Farr assay, whereas at the time of the diagnosis, the immunofluorescence on *Crithidia luciliae* immunofluorescence test (CLIFT) or the Farr assays may offer the best specificity.<sup>20</sup> Discrepancies between clinical assessment and laboratory results have also been reported. It is our recommendation that in cases of high clinical suspicion a physician request for the determination of specific antibodies should be granted, irrespective of the result of previous tests. For instance, specific antibodies directed against Jo-1, ribosomal P or SS-A/Ro may be detected in patients who are ANA negative by IIFA. In this regard, one may consider the possibility that for an individual patient serology may change over time. Another important issue at present concerns the use of ANA/anti-DNA testing as inclusion criteria for clinical trials that may require the use of several platforms balancing specificity versus sensitivity.

## Recommendation

**Table 2** IIFA nuclear/cytoplasmic patterns detected on HEp-2 substrates and related antigens/diagnosis

Most commonly recognised patterns		
Nuclear patterns	Related antigens	Related diagnosis
Homogeneous	<b>dsDNA, histones, chromatin/nucleosomes</b> , HMG	SLE, drug induced SLE/vasculitis, JIA
Coarse speckled	<b>U1-SnRNP, U2-6 snRNP (5m)</b> , nuclear matrix	MCTD, SLE, Raynaud, SSC, SS, UCTD
Fine speckled	<b>SSA/Ro, SSB/La, Topo-1</b> , common to many antigens	SLE, SS, SSC, IM, MCTD
Centromere	<b>Kinetochore: CENP-A, B, C, F</b>	SSc (limited), Raynaud's
Nucleolar	<b>PM/Scl</b> , RNA-polymerase, URNP, U3-RNP, To/Th, B23 phosphoprotein/numatrin	SSc, Raynaud's, IM, overlap
Cytoplasmic patterns		
Diffuse	<b>RibP, Jo-1</b> , other tRNA synthetases, SRP	SLE, IM
Fine speckled	<b>Jo-1, SRP, PDH (mitochondria)</b>	IM, DM, PBC, interstitial lung disease
Less commonly recognised patterns		
Nuclear patterns	Related antigens	Related diagnosis
Peripheral/rim or nuclear envelope	Lamins, LAP1/2 gp210, nucleoporin p62; nuclear envelope and nuclear pore complex antigens	SLE, RA, PBC, IM autoimmune liver diseases
Dense fine speckled	<b>DFS70/LEDGF-P75</b>	Healthy subjects and other inflammatory conditions
Pleomorphic cell cycle speckled (PCNA)	Auxiliary protein proliferating cell nuclear antigen: elongation factor of DNA polymerase delta	SLE, lymphoproliferative diseases, SS
Nucleolar (clumpy)	<b>U3-SnRNP (fibrillarin)</b>	SSc
Multiple/few nuclear dots	<b>Sp100, PML bodies, p80-coilin</b>	PBC, CAH, SS
Centrosome/centriole (formerly:spindle apparatus)	<b>Enolase, ninein, pericentrin</b>	SSc, Raynaud's, inflammatory disease
MSA (mitotic spindle)	<b>NuMA/centrophilin</b>	RA, inflammatory conditions; pneumonia (mycoplasma)
Cytoplasmic patterns		
Discrete speckled	<b>Endosome (early endosome antigen 1), GW/Processing bodies, multivesicular bodies/lysosomes</b>	Neurological conditions, SS, SLE, RA, PBC, UCTD
Golgi complex	<b>Golgi proteins/golgins: giantin, golgin 245, golgin 110, golgin 97, golgin 95, others</b>	SLE, SS, RA, overlap syndromes, cerebellar ataxia
Cytoplasmic fibres	<b>Actin, cytokeratin, tropomyosin, vimentin</b>	CAH, DM, infections and other inflammatory diseases

Within the many patterns that can be distinguished the ones specified in the upper part of the table are the most commonly recognised. The relationship between pattern and antigen specificity may differ in certain conditions. Similarly, the specific antigens marked in bold are the most commonly detected by reflex testing, although other antigens may be of importance in different clinical conditions. Less common pattern are specified in the lower part of this table.

CAH, chronic autoimmune hepatitis; CENP, centromere protein; DFS, dense fine speckled; DM, dermatomyositis; dsDNA, double-stranded DNA; GW, glycine—tryptophan; HMG, high mobility group; IM, inflammatory myopathies; JIA, juvenile idiopathic arthritis; MCTD, mixed connective tissue disease; MSA, mitotic spindle; NuMA, nuclear mitotic apparatus; PBC, primary biliary cirrhosis; PDH, pyruvate dehydrogenase; PM/Scl, polymyositis/scleroderma; RA, rheumatoid arthritis; RibP, ribosomal P protein; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SRP, signal recognition particle; SS, Sjögren's syndrome; SSC, systemic sclerosis; Topo-1, topo-isomerase 1; tRNA, transfer RNA; UCTD, undifferentiated connective tissue disease.

### Communication between the laboratory bench and the clinic

This is an all-embracing issue detailed in many of our recommendations (ie, 7, 11, 12, 13, 14, 16, 19, 20 and 21). Notably, many clinicians are unaware of the specific method or the differences between methods used for ANA detection, and may erroneously apply the test characteristics of IIFA to other methods. Therefore, by and large the autoimmune laboratory should specify each method used. In addition, in the case of ANA detection by IIFA, titres and patterns obtained should be reported. If multiple methods are being used either for ANA, anti-dsDNA or other antigen-specific autoantibodies, the results obtained by each method should be reported separately. In addition, if further testing is advised (eg, the determination of anti-dsDNA in samples with high titre homogenous ANA by IIFA) a laboratory comment may be included as detailed below.

### The recommendations

The first subgroup of recommendations (1–13) is dedicated to ANA screening at the first level of diagnosis.<sup>1</sup> Most of our

knowledge regarding ANA screening is based on data obtained by IIFA. As such, the prevalence of ANA–IIFA among SLE patients is expected to be above 95%, while a lower prevalence may be detected using methods that rely on a limited number of antigens.<sup>7–8</sup> Furthermore, ANA can be detected in patients with autoimmune, malignant or infectious diseases as well as in healthy subjects. Hence, the performance of any ANA assay is highly dependent on its pre-test probability, and interpretation of results should be governed by clinical circumstances.<sup>1–18</sup> In these recommendations, like others, we have recognised IIFA on HEp-2 (or HEp-2000) to be the reference method of choice for ANA screening.<sup>6–21</sup> However, we have also acknowledged the limitations of IIFA, including the time required for its performance and the necessity of highly qualified laboratory personnel. The latter raises the urgent need for training programmes on IIFA performing and autoimmune serology interpretation.<sup>22</sup> In contrast, the alternative automated methods (see supplementary material, available online only) have come into common use, especially in high-throughput laboratories. These methods are



simple to operate and may have the added value of better recognition of specific antibodies. Alas, they often lack sufficient sensitivity for certain diseases (ie, SLE and SSC)<sup>23–27</sup> (recommendation 5). Considering the pro and cons, we have included the alternative assays in our recommendations as long as the platform being used is clearly specified (recommendation 6) and the result is not referred to as ‘ANA test’ or ‘ANA screen’, which are typically associated with ANA testing by IIFA (recommendation 7).

From the laboratory perspective, standardisation of IIFA is vital, and several variables of this method have been specified. The recommended substrate for ANA–IIFA derives from a human epithelial cell line (HEP-2 or HEP2000) and should contain a sufficient number of mitotic cells to enable adequate pattern recognition. Low positive control samples as well as an internal cut-off control, randomly distributed in distinct runs, may enable continuous training of technicians. The secondary detection antibody (conjugate) should consist of fluorochrome-labelled anti-human IgG-specific antiserum (recommendation 9), either FITC or another validated new-generation fluorochrome. A special consideration, at a regular interval, to the fluorochrome potency and ultraviolet bulb strength was suggested. Another variable of ANA testing is the screening dilution of sera.<sup>6 21</sup> Following review of the literature and many discussions among the committee experts, it was agreed that the optimal screening dilution must be defined locally, and the titre related to the 95th percentile of healthy controls considered the optimal screening dilution. In cases of uncertainty, while taking into consideration sensitivity, specificity and cost (ie, wells on slides, technician time, other reagents), we have pointed out that a screening dilution of 1 : 160 was often found to be the most suitable for the evaluation of adult patients.<sup>6 21</sup> In an international study, ANA was documented in 31.7% of healthy subjects at 1 : 40 serum dilution, 13.3% at 1 : 80 and 5.0% at 1 : 160.<sup>21</sup> Conversely, at 1 : 160 dilution the sensitivity for SARD is not perfect and a negative result does not exclude disease. Notably, no consensus could be achieved among experts regarding the screening dilution for children (<16 years), for which some use a dilution of 1 : 40, or the need for further dilution once a positive result was obtained. Previously, it was generally accepted that individuals without autoimmune disease would present with lower serum levels while high-titre ANA are clinically more significant.<sup>12</sup> However, there is ample evidence that low-titre ANA may be significant and that titres following the screening threshold of 1 : 80 or 1 : 160 have no bearing on diagnosis or disease activity.<sup>7 15</sup>

Patterns of ANA by IIFA reflect the topographic distribution of target autoantigens (recommendations 11, 12)<sup>28</sup> and may convey significant information about antibody specificity (table 2).<sup>29</sup> The role of cell cycle dynamics (ie, interphase, mitotic) in defining patterns is of great importance, especially for complex patterns related to autoantigens that exhibit a dynamic behaviour along the cell cycle, such as centromere protein (CENP)-F, NuMA-1 and topoisomerase-1.<sup>30–32</sup> The recognition of both nuclear and cytoplasmic patterns enables ‘reflex testing’ (ie, testing for specific antibodies depending of the pattern) and improves the utility of the serological evaluation (recommendation 13).<sup>1 28</sup>

Antibodies directed against dsDNA are a key marker for diagnosis and follow-up of patients with SLE<sup>33 34</sup> (recommendations 14–18). Theoretically, if ANA–IIFA is negative one should not proceed to defining anti-dsDNA antibodies,<sup>35 36</sup> although if clinical suspicion of SLE is substantial anti-dsDNA antibodies assessment may be requested by the clinician. On the other hand, certain IIFA patterns (eg, homogenous) are suggestive of anti-dsDNA antibody (table 2).<sup>37</sup> Regrettably, in most laboratories ‘reflex testing’ cannot be performed unless requested;

thereby the addition of a note to the ANA–IIFA result should be considered (eg, ‘This pattern may represent antibodies directed to DNA or chromatin components. Further tests are advised if there is a clinical suspicion of systemic lupus erythematosus’). The appropriate method of anti-dsDNA antibodies determination is still a matter of debate. The Farr assay and the CLIFT offer high clinical specificity (recommendation 15).<sup>35 38</sup> In point of fact, the Farr assay is the one ‘specific’ for anti-dsDNA related to SLE diagnosis, owing to its ability to detect high avidity antibodies. However, both assays have substantial drawbacks. The Farr assay requires the use of radioisotopes, and may detect anti-dsDNA antibodies induced by drugs (ie, anti-tumour necrosis factors)<sup>39</sup> while the CLIFT is not quantitative and is accompanied by relatively low sensitivity. Alternative methods for anti-dsDNA detection have recently been developed. Most of them provide high sensitivity but are hampered by lower specificity and lack of correlation with each other.<sup>38 40</sup> Reflecting on these aspects, we have recommended that positive results obtained by these alternative methods be confirmed by CLIFT or Farr assay. Nonetheless, if discrepancy between methods is observed both results should be reported. In such cases a careful explanation should be provided by the laboratory and the final interpretation should take into consideration the clinical scenario and the ANA–IIFA pattern. Last but not least, for follow-up of anti-dsDNA titres a quantitative method (ie, not CLIFT) (recommendations 16, 17), preferably by the same method and performed by the same laboratory, is recommended (recommendation 18).

The identification of antibody specificity to nuclear and cytoplasmic antigens may further differentiate between diseases<sup>41–44</sup> (table 2; recommendation 19). Although there is a clear correlation between IIFA patterns and antigen specificity (table 2), this association is not perfect and antigen specificity cannot be deduced from the IIFA pattern alone. Furthermore, disagreements between methods to detect specific antibodies and/or IIFA patterns were reported.<sup>44–46</sup> In these cases an alternative method can be employed (recommendations 1, 20, 21). In addition, unlike anti-dsDNA, the coexistence of negative ANA by IIFA and seropositivity for specific antibodies by solid phase assay is not rare (ie, anti-SSA/Ro).<sup>47</sup> Thus, a physician request to determine specific antibodies should be granted, irrespective of ANA–IIFA (recommendation 23). Notably, another aspect concerning anti-SSA/Ro antibody is the detection of its variants directed at Ro60 and Ro52/TRIM21, as some tests currently in use include only the Ro60 antigen. Detection of anti-SSA/Ro is recommended in different scenarios including counselling of patients with autoimmune disease who desire a pregnancy. The latter are mainly related to anti-Ro52/TRIM21 antibodies. Therefore, specifying the antigen used and evaluation of both anti-Ro52 and anti-Ro60 should be considered. The importance of specific antibody levels is still a matter of debate. In particular, the significance of anti-ribonucleoprotein levels (recommendation 22) data are lacking regarding levels determined by current methods in comparison to the historical haemagglutination test used for the mixed connective tissue disease criterion.<sup>48</sup>

The fourth subgroup of our recommendations addresses the issue of local validation of methods (24, 25). Both recommendations were fully accepted by the IUIS group and significantly less by the EASI teams. This divergence between experts may be explained by the different regulations applied in the different countries, namely in America and Canada local validation is frequently required by the authorities. Nevertheless, as the list of available methods is bound to increase, it is highly likely that in the years to come verification of assays will be an important feature of autoimmune laboratories worldwide.

## Recommendation

### CONCLUSIONS

Defining anticellular antibodies is an essential component of the evaluation of patients with autoimmune diseases. The wide array of methods for their determination prompted questions and debate among laboratory personnel, clinicians, as well as co-authors of this manuscript. Therefore, in this study we have offered recommendations that are applicable and may facilitate the design of local algorithms for ANA detection. We have pointed out that the changes in the field of autoimmune diagnostics necessitate dedicated training of those who demand, perform and interpret the results. Therefore, in parallel to defining new autoantibodies and the development of new platforms an urgent need for training programmes for clinician, technicians and the industry has been recognised. Last but not least, further studies are required to address issues such as the local validation of platforms, significance of titres and patterns among different populations (ie, children), and different stages of diseases (ie, pre-clinical vs fully established; active vs quiescent) and the role of novel automated IIFA methods that may overcome many limitations of the manual form of this assay.

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**Contributors** All authors have actively participated in this study as well as in writing the manuscript.

**Competing interests** None.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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## International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies

Nancy Agmon-Levin, Jan Damoiseaux, Cees Kallenberg, et al.

*Ann Rheum Dis* published online October 14, 2013

doi: 10.1136/annrheumdis-2013-203863

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